Saskatchewan Ministry of Agriculture

RESEARCH

FINAL REPORT

ADF

AGRICULTURE DEVELOPMENT FUND

20040567

RESISTANCE TO SCLEROTINIA SCLEROTIORUM (STEM ROT) IN BRASSICA NAPUS (CANOLA)

Funded by: The Agriculture Development Fund

March 2009

Prepared by: Agriculture & Agri-Food Canada (AAFC)

Final Project Report to ADF

Date: March 15, 2009

Reporting Period: Final Report

Investigators: Dwayne Hegedus, Roger Rimmer and Lone Buchwaldt

Project Title: A Genomics Approach to Sclerotinia Resistance in *Brassica napus*

Executive Summary

The long term objective of the project is to provide the canola industry with specific resistance genes and molecular markers linked to resistance for developing stem rot resistant *Brassica napus* cultivars. In the short term, our objectives were: 1) to identify candidate resistance genes using genomics methods, such as expressed sequence tags (ESTs) and gene arrays, and to examine the contribution of select genes to resistance; and 2) to develop a better understanding of the factors employed by the fungus to cause disease and to determine how the plant inactivates these factors.

This project built upon the resources and information developed in a previous project (2000-2004) funded by Saskatchewan Agriculture and Food, the Canola Council of Canada, the Saskatchewan Canola Development Commission and the AAFC Matching Investments Initiative. An allied project funded by an international industry consortium was established at AAFC-SRC that will develop molecular markers linked to quantitative trait loci for sclerotinia resistance for marker-assisted selection in breeding programs; this effectively linked federal, provincial government, producer and industry resources in a concerted research effort to overcome stem rot disease in canola. Markers are essential to introduce resistance genes into suitable canola breeding lines. Resistance genes identified using genomics approaches have contributed to the development of these markers, and vice versa.

The winter-type *B. napus* Zhong You 821 (ZY821) developed in China is one of the best cultivars for resistance to stem rot (Figure 1). At AAFC, we employed genomics approaches to identify the genes responsible for sclerotinia resistance in ZY821. Using powerful techniques, such as cDNA subtraction and DNA micro-arrays, we identified the suite of genes expressed in ZY821 in response to *S. sclerotiorum* infection. In a parallel project, doubled haploid (DH) lines

from several resistant ZY821 x susceptible *B. napus* crosses (mapping populations) were developed, and we are using this material to identify genes expressed specifically in resistant but not in susceptible lines. A select number of putative resistance genes were introduced into a susceptible *B. napus* line to determine their individual contribution to resistance. Several other genes identified in the cDNA subtraction and DNA microarray experiments are being mapped to loci with resistance which will facilitate introgression of the resistance genes into elite canola lines.

The second component of the project examined how *S. sclerotiorum* causes disease and the mechanisms used by the plant to prevent this. In this regard, five polygalacturonases (enzymes that break down pectin) and two necrosis-inducing proteins were characterized and found to be the principle factors responsible for the development of necrotic lesions. Using a genomics approach, the genes encoding 17 *B. napus* polygalacturonase inhibitor proteins were identified and characterized. At least three of these was found to inhibit the activity of *S. sclerotiorum* polygacturonases and are being evaluated as a potential resistance gene candidate.

Technical Report:

In cases where the work arising from the project was published, the technical report provides only a summary of the most important findings. Additional detail is provided in the publications and appendices associated with this document which are listed at the beginning of the appropriate sections.

Objective I: Identification of genes associated with resistance in *B. napus* ZY821
Subproject A: Examining Gene Expression Patterns

See the following appendices for additional details:

Zhao J., Buchwaldt, L., Rimmer R., Sharpe A., Bekkaoui D, Hegedus DD. (2009) Temporal and qualitative differences in gene expression underly the response of resistant and susceptible *Brassica napus* cultivars to *Sclerotinia sclerotirum*. Mol. Plant Pathol. – in press.

Zhao, J., Buchwaldt, L., Rimmer, S.R., Brkic, M., Bekkaoui, D. and Hegedus, D.D. (2009) Differential expression of duplicated peroxidase genes in the allotetraploid *Brassica napus*. Plant Physiol. Biochem. – in press.

Supplementary Table S1. List of differentially expressed genes in *B. napus* cv. Westar
Supplementary Table S2. List of differentially expressed genes in *B. napus* cv. ZY821
Supplementary Table S3. Comparison of inoculated *B. napus* cv. ZY821 and Westar

I. Patterns of gene expression in susceptible and resistant *B. napus* lines

To simulate natural infection of *B. napus* stems by *S. sclerotiorum* under field conditions, we attached mycelial plugs to the stems of flowering plants. Visible lesions developed within 24 - 48 hours post inoculation (hpi). After 7 days, lesions on stems of the resistant cultivar ZY821 were smaller than on the susceptible cultivar Westar, and most Westar plants died within 21 days of inoculation (Figure 1).

Identifying the induced defense genes was an important first step toward understanding sclerotinia resistance. Initially, we developed a subtractive cDNA library from infected vs non-infected stems of the resistant *B. napus* line ZY821 and identified 76 genes that were expressed in response to *S. sclerotiorum* inoculation. A subset of these genes was found to be highly induced in response to *S. sclerotiorum* inoculation, including those encoding anti-fungal proteins such as the pathogenesis-related proteins (PR) PR1 and PR5, chitinase (5 types), endoglucanase and two lectins (Figure 2). However, it was equally likely that resistance was due to alterations in the timing of induction (i.e. earlier) and/or to higher levels of expression of these defenses, which would be determined by a small number of regulatory proteins. Members of gene families, such as regulatory proteins, are generally under-represented in subtractive cDNA libraries since they share a high degree of DNA sequence similarity. To overcome this problem, we used a *B. napus* oligonucleotide microarray representing 15,000 unique genes to study the response in stems to inoculation with *S. sclerotiorum* in resistant and susceptible cultivars. The micro-array was designed by AAFC and was the most comprehensive *B. napus* array available at that time.

A large number of genes were found to be differentially regulated after infection (Figure 3 and 4). The gene expression profiles during the latter stages of stem infection were similar to that of *B. napus* seedlings in response to *S. sclerotiorum* infection (Yang *et al.*, 2007; Zhao *et al.*, 2007) and other necrotrophic pathogen-host interactions, namely *Botrytis cinerea - A. thaliana* and *Alternaria brassicicola - Brassica oleracea*. Our sampling was biased toward the very early stages of the infection since our hypothesis was that events occurring soon after the initial interaction between pathogen and host were critical for resistance to aggressive necrotrophs such as *S. sclerotiorum*. We chose to examine infected stems since this represented the natural site for infection in this pathosystem. Indeed, we observed that the resistant line, ZY821, induced defense-associated genes sooner than the susceptible line, Westar. We also observed differences in the expression patterns of a large group of regulatory genes, as well as genes involved in plant hormone synthesis and aspects of the host defense mechanisms against pathogens. Changes in the expression of genes involved in carbon metabolism suggest that carbon storage reserves (such as sucrose, starch and lipid) are accessed and shuttled through the photorespiration pathway. This pathway leads to the formation of glyoxylate that can enter the TCA cycle, as well

as hydrogen peroxide that may form a part of the defense response. The activity of the TCA cycle may be greatly increased as evidenced by the induction of many genes encoding TCA cycle enzymes. This not only generates reducing power and energy, but also precursors for amino acid biosynthesis. We observed a sharp increase in transcript abundance for genes encoding enzymes for tryptophan biosynthesis, this amino acid being a precursor for both glucosinolates and phenylpropanoids. This study has provided new insight into aspects of the defense response to necrotrophic pathogens using an experimental design that closely models the actual circumstances occurring in the field. We are currently exploring several targets to determine their contribution to resistance in *B. napus* ZY821 and other resistant cultivars (see Subproject C).

To further these studies, three individual spring-type resistant and three susceptible DH lines from a *B. napus* ZY821 X Westar cross (Figure 5) were identified for comparison and subsequent identification of unique resistance genes or patterns of expression associated with resistance.

II. Effect of polyploidization on the expression and function of *B. napus* defense genes

Gene redundancy due to polyploidization provides a selective advantage for plant adaptation. In the context of the response to the fungal pathogen *S. sclerotiorum*, we examined the expression patterns of two peroxidase genes (*BnPOX1* and *BnPOX2*) in the natural allotetraploid *B. napus* and the model diploid progenitors *Brassica rapa* (Br) and *Brassica oleracea* (Bo). We demonstrated the Bo homeolog of *BnPOX1* was up-regulated after infection, while both *BnPOX2* homeologs were down-regulated. A bias toward reciprocal expression of the homeologs of *BnPOX1* in different organs in the natural allotetraploid of *B. napus* was also observed. These results suggest that subfunctionalization of the duplicated *BnPOX* genes after *B. napus* polyploidization, as well as subneofunctionalization of the homeologs in response to this specific biotic stress has occurred. Retention of expression patterns in the diploid progenitors and the natural allotetraploid in some organs indicates that the function of peroxidase genes was conserved during evolution. This study is important as it is now apparent that the function of some genes in diploid progenitors may not be retained in the polyploid derivatives. This will

have to be taken into consideration when evaluating the introgression of genes from wide crosses and resynthesized polyploid lines.

Subproject B: Relationship between ESTs and Quantitative Trait Loci (QTL) for Resistance

In an allied project (investigators: Buchwaldt, Rimmer and Lydiate) quantitative trait loci (QTL) conferring resistance to sclerotinia stem rot in ZY821 are being mapped. Several populations of doubled haploid lines and recombinant inbred lines were developed from crosses with the resistant line into both spring type and winter type back grounds. The progenies were genotyped with microsatellite markers and phenotyped by inoculation of stems with *S. sclerotiorum* (isolate #321). QTL analysis by composite interval mapping (QTL Cartographer) of the combined data set has identified six QTL in spring type populations and four in winter type populations. The most significant QTLs mapped to linkage groups N5, N6, N7, N16 and N19 with LOD values between 7 and 10 and phenotypic variability explained between 19 and 43 %. We are in the process of determining if these QTL correspond to the map location of ~20 defence related genes which were significantly up regulated in ZY821 during early stages of the infection process as evident from our microarray study. First we utilize the high degree of similarity between the *Arabidopsis thaliana* and *B. napus* genomes to determine the approximate map location of each defense gene. We then develop PCR based primers and screen selected DH or RIL in order to determine if they co-segregate with the molecular markers linked to each QTL.

Subproject C: Selection and testing candidate genes for resistance to stem rot

See the following appendices for additional details:

Hegedus DD, Li, R., Buchwaldt L., Parkin I., Whitwill S., Coutu C., Bekkaoui D, Rimmer SR. (2008). *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially-regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defence hormone treatment. Planta 228:241-253.

Li, R., Rimmer, R., Yu, M., Sharpe, A.G., Séguin-Swartz, G., Lydiate, D. and Hegedus, D.D. (2003) Two polygalacturonase inhibitory protein genes are differentially expressed in response to biotic and abiotic stresses in *Brassica napus*. Planta 217: 299-308.

I. *B. napus* Polygalacturonase Inhibitory Proteins (PGIP)

Plants encode a distinct set of PGIP that function to inhibit polygalacturonase enzymes produced by soft rot fungal pathogens. Initially, we characterized two PGIP-encoding genes (*Bnpgip1* and *Bnpgip2*) from *B. napus* DH12075 (a doubled haploid line derived from a cross between 'Crésor' and 'Westar'). The two proteins exhibited 67.4% identity at the amino acid level and contained 10 imperfect leucine-rich repeats. The *pgip* genes appeared to be members of a small multigene family in *B. napus* with up to 4 members. *Bnpgip1* and *Bnpgip2* were constitutively expressed in root, stem, flower bud and open flowers. In mature leaf tissue, different levels of induction were observed in response to biotic and abiotic stresses. *Bnpgip1* expression was highly responsive to flea beetle feeding and mechanical wounding, weakly to *S. sclerotiorum* infection and exposure to cold but not to dehydration. Conversely, *Bnpgip2* expression was strongly induced by *S. sclerotiorum* infection and to a lesser degree by wounding but not by flea beetle feeding. Application of jasmonic acid to leaves induced both *Bnpgip1* and *Bnpgip2* gene expression; however, salicylic acid did not activate either gene. Taken together, these results suggested that separate pathways regulate *Bnpgip1* and *Bnpgip2* and their role in plant development or resistance to biotic and abiotic stress differs. The two cDNA fragments were used separately to screen a *B. napus* DH12075 bacterial artificial chromosome (BAC) library. BAC clones with unique restriction digest patterns were sequenced and a set of at least 17 PGIP genes similar to *Bnpgip1* or *Bnpgip2* were discovered. This is the largest *pgip* gene family reported to date. Comparison of the BnPGIPs revealed several sites within the xxLxLxx region of leucine rich repeats that form β-sheets along the interacting face of the PGIP that were hypervariable and represent good candidates for generating PGIP diversity. Characterization of the regulatory regions and RT-PCR studies with gene-specific primers revealed that individual genes were differentially responsive to pathogen infection, mechanical wounding and signaling molecules. Many of the *Bnpgip* genes responded to infection by the necrotic pathogen, *S. sclerotiorum*; however, these genes were also induced either by jasmonic acid, wounding and salicylic acid or some combination thereof. The large number of PGIPs and the differential manner in which they

are regulated likely indicates that *B. napus* has an inherent ability to respond to attack from a broad spectrum of pathogens and pests.

II. Interaction of *S. sclerotiorum* polygalacturonases with *B. napus* PGIP

A graduate student was employed to determine which of BnPGIPs inhibit the activity of *S. sclerotiorum* polygalacturonases (SSPG). The secretory yeast *Pichia pastoris* was transformed with the vector, pPICZa, containing cassettes designed to express and secrete hexahistidine-tagged SsPG1, SsPG2, SsPG3, SsPG5, SsPG6 as well as BnPGIP1, BnPGIP2, BnPGIP7 and BnPGIP16 in a methanol inducible manner. PGIP1 was expressed at the highest level of all the PGIPs. PGIP7 and PGIP16 were expressed at somewhat lower levels and PGIP2 expression was undetectable in either of lines. PGIP1 concentration increased in the expression media over a period of six days and was detectable by western blot analysis but was not visible after separation on an SDS-PAGE gel followed by staining with Coomassie Blue (Figure 6). The bulk of the PGIP1 was secreted and not associated with the cell; however, expression levels were low requiring concentration followed by purification prior to conducting biochemical analyses. This involved concentration using dialytic membranes, ammonium sulphate precipitation or trichloroacetic acid/acetone precipitation followed by purification on a nickel affinity chromatography column.

Of the five SSPGs examined, SSPG3 and SSPG6 were expressed at very high levels (Figure 6). The level of SSPG5 was not sufficient to be detected by staining with Coomassie blue after SDS-PAGE separation but was detectable by western blot analysis. These SSPGs were concentrated successfully using Centricon tubes. SSPG1 expression was not detectable by western blot analysis of neat culture supernatant, but was detectable after concentration of the samples. The SSPGs were shown to possess pectinolytic activity (Figure 7).

In order to characterize the biochemical requirements for the individual PGs and to examine their interaction with PGIPs it was necessary to develop a consistent assay to quantify PG activity. Dinitrosalisylic acid (DNSA) interacts with free galacturonic acid (GA) released by PG activity on pectin to form a colored compound that can be quantified using a spectrophotometer. Several modifications of the existing protocol were required to stabilize the readings and make the assay

more robust. PG-PGIP interaction studies were conducted with the concentrated PGIPs and SSPG3 and SSPG6. PGIP1 and PGIP7 were found to inhibit SSPG6 and PGIP16 was found to consistently inhibit the activity of SSPG3 (Figure 8).

III. Testing of Candidate Resistance Genes in *B. napus* and *A. thaliana*

We have begun evaluating candidate resistance genes by expressing them in the susceptible *B. napus* cultivar DH12075 and the model plant *A. thaliana*. The initial set of candidate genes was selected from the earlier subtractive cDNA library and included a highly induced dual function lectin-chitinase (hevein) and two proteins (BnPGIP1 and BnPGIP2) that inhibit the activity of pathogen enzymes needed to destroy plant tissues. The constructs were designed to over-express the genes constitutively (present in all tissues all the time) or to express them only in the stem in response to sclerotinia infection (using an inducible promoter from one of the *Bnpgip* genes above). Homozygous transformed lines were identified and are being rated for disease resistance. Initial results indicated that constitutive expression of *B. napus* hevein 4 increased resistance; this observation is now being confirmed.

Oxalate oxidase breaks down the oxalic acid released by the pathogen and has been shown to provide resistance to *S. sclerotiorum* in soybean (D. Simmonds, pers. comm.). We evaluated *B. napus* lines expressing wheat oxalate oxidase and did not observe an increase in resistance. Therefore, we generated *A. thaliana* lines expressing a wheat oxalate oxidase gene, either alone or in combination with *BnPgip1* or *BnPgip2*. This material is currently being evaluated.

A second set of candidate genes was derived from the micro-array experiments comparing resistant and susceptible lines in subproject A. One of these was a WRKY-type transcription factor that has been implicated in inducing defense response in other plants. Genes encoding three WRKY factors, BnWRKY33, BnWKY40 and BnWKY53 (named according to the most closely related *A. thaliana* ortholog), were induced within 6-12 hpi in ZY821. As these may be important for regulating the expression of genes encoding defense proteins, we examined the signaling pathways required for induction of *BnWRKY33* gene expression since it was associated with a QTL marker for *S. sclerotiorum* resistance in *B. napus* (Zhao and Osborn, 2007). Treatment of young leaf tissue with salicylic acid (an inducer of system acquired resistance

genes) induced expression within 12 h of *BnWRKY33* whereas jasmonic acid and the ethylene precursor, ACC, had little if any effect (Figure 9). Two other candidate genes were over-expressed in *B. napus*. These encode a concanavalin-like lectin, the expression of this gene was up-regulated 20,000 fold after *S. sclerotiorum* infection, and annexin, a protein that is involved in cytoskeleton organization and believed be involved in the delivery of defense proteins to the site of infection. Homozygous, single insert lines are currently being identified for subsequent evaluation of disease resistance.

Objective II: *S. sclerotiorum* genes associated with pathogenesis

See the following appendices for additional details:

Dallal Bashi, Z, Rimmer, SR, Buchwaldt, L, Khachatourians GG and Hegedus DD. (2009) Factors affecting the concerted expression of *Sclerotinia sclerotiorum cutinaseA* and *polyglacturonase1*. (draft manuscript).

Dallal Bashi, Hegedus, D.D., Buchwaldt, L., Rimmer, S.R. and Borhan, M.H. (2009) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene inducing peptides (NEPs). Mol. Plant Pathol – in press.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A.G., Séguin-Swartz, G., and Hegedus, D.D. (2004a) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: Cloning and characterization of endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A.G., Séguin-Swartz, G., and Hegedus, D.D. (2004b) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* cultivar: Expressed sequence tag analysis identifies genes associated with fungal pathogenesis. Fungal Genet. Biol. 41: 735-753.

I. Genes expressed during *S. sclerotiorum* gene infection.

Initially, we identified *S. sclerotiorum* genes expressed during infection (or a condition that simulated infection) through EST analysis (Li et al. 2004a, b). To identify genes involved in fungal development and pathogenesis we generated 2232 ESTs from two cDNA libraries

constructed using either mycelia grown in pectin medium or tissues from infected *B. napus* stems. A total of 774 individual fungal genes were identified of which 39 were represented only among the infected plant EST collection. Annotation of 534 unigenes was possible following the categories applied to *Saccharomyces cerevisiae* and the Universal Gene Ontology scheme. cDNAs were identified that encoded potential pathogenicity factors including four endopolygalacturonases, two exopolygalacturonases and several metabolite transporters. A non-redundant set of *S. sclerotiorum* ESTs was deposited in Genbank (Accession CD645592-CD646371, CF542256-CF542258, CF602382-CF602389) and COGEME (cogeme.ex.ac.uk) databases.

II. Isolation of genes encoding *S. sclerotiorum* polygalacturonases (PG)

Five major and several minor PG isoenzymes were identified in a *S. sclerotiorum* isolate from *B. napus* by isoelectric focusing and pectin gel overlays. Using a combination of degenerate PCR and expressed sequence tags (EST) four endo-PG genes, designated as *sspg1d, sspg3, sspg5 and sspg6*, and two exo-PG genes, *ssxpg1* and *ssxpg2*, were identified. SSPG1d is a member of a previously described PG gene family. The mature SSPG1d is a neutral PG, whereas fully processed SSPG3, SSPG5 and SSPG6 are acidic enzymes.

III. Characterization of *S. sclerotiorum* polygalacturonases and cutinase

Under saprophytic growth conditions, *sspg1d*, *sspg3*, *sspg5* and *ssxpg1* expression was induced by pectin and galacturonic acid and subject to catabolite repression by glucose. Conditions could not be identified under which *sspg6* or *ssxpg2* were expressed well. Transfer of mycelia from liquid media to solid substrates induced expression of *sspg1d* suggesting that it may also be regulated by thigomotrophic interactions. Under pathogenic conditions, *sspg1d* was highly expressed during infection. *sspg3* was also expressed during infection, albeit at lower levels than *sspg1d*, whereas *sspg5*, *sspg6* and *ssxpg1* were expressed only weakly.

A graduate student, who has recently switched to a PhD program, was employed to examine the contribution of these enzymes to fungal virulence using gene inactivation systems (gene disruption and anti-sense) recently developed for *S. sclerotiorum*. During host plant infection, *S. sclerotiorum* releases hydrolytic enzymes that sequentially degrade the cuticle, middle lamellae,

primary and secondary cell walls. We examined the role of the plant cuticle in *S. sclerotiorum* infection and the interplay between physical contact perception and carbon metabolism on *sspg1* expression. We also examined the factors affecting the regulation of the genes encoding SSPG1 (*sspg1*) and a newly identified cutinase (*sscutA*). *In vitro*, *sscutA* transcripts were detected within 1 hour after inoculation of leaves and its expression was primarily controlled by mycelial contact with solid or semi-solid surfaces including the leaf. Expression of *sspg1* was also induced by contact with solid surfaces including the leaf, although only slightly. Its expression was mainly restricted to actively growing mycelia at the expanding margin of the lesion. *sspg1* expression was induced by pectin at low pH and was repressed by elevated levels of galacturonic acid. We show that glucose supported either a basal level of expression, but accentuated expression when provided to mycelia used to inoculate leaves which is contrary to earlier reports that it repressed *sspg1* expression. Calcium signaling and cellular cAMP levels were involved in the regulation of *sscutA* and *sspg1*. Blocking of calcium channels or elevating intracellular cAMP levels abolished expression of *sscutA* and *sspg1* and decreased *S. sclerotiorum* pathogenicity.

IV. *S. sclerotiorum* factors contributing necrosis

Successful host colonization by necrotrophic plant pathogens requires induction of plant cell death to provide nutrients needed for infection establishment and progression. The endo-PGs SSPG3 and SSPG6 caused the formation of necrotic lesions when infiltrated into leaves or when placed on leaves with an abraded cuticle. Leaves that had been stripped of cuticle were more effectively colonized by *S. sclerotiorum*.

We have also cloned two genes encoding necrosis and ethylene inducing peptides from *S. sclerotiorum* which we named *SsNep1* and *SsNep2*. Both peptides induce necrosis when expressed transiently in tobacco leaves. *SsNep1* is expressed at a very low level relative to *SsNep2* during the infection. The expression of *SsNep2* was induced by contact with solid surfaces and was expressed in both the necrotic zone and at the leading margin of the infection. *SsNep2* expression was dependent on calcium and cyclic AMP (cAMP) signaling as compounds affecting these pathways reduced or abolished *SsNep2* expression coincident with partial or total loss of virulence. An adenylate cyclase deletion mutant exhibited a similar phenotype.

V. Regulation of virulence gene expression

To better understand the signaling pathways involved in regulating the production of virulence determinants we are studying two key types of mitogen-activated protein (MAP) kinases, Bmp1 and Mkc1, that have been implicated in transduction of the surface sensing signal in *Candida albicans* and *Botrytis cinerea*, respectively. Such MAP kinases are expected to play a similar role in the response of *S. sclerotiorum* to different surfaces. *Bmp1* expression was regulated by both carbon source and surface contact (Figure 9). Transfer of mycelia to normal or wax-stripped leaves had no effect on expression; however, levels were noticeably higher in the absence of glucose suggesting it is partially regulated by catabolite repression. Conversely, transfer to Parafilm strongly repressed expression of *Bmp1*. Similarly, *Mkc1* was also repressed by contact with Parafilm (Figure 11). On the other hand, transfer to the surface of intact leaves induced *Mkc1* expression only in the presence of glucose, which was similar to that observed for *SsPG1*. Mutants where *SsMk1* gene was disrupted were avirulent indicating that we have identified a major signaling pathway required for the expression of pathogenicity genes.

Personnel:

Name	Position/Level	Source	Time
Diana Bekkaoui	Technician	ADF	100 %
Zafer Dallal Bashi	Graduate Student	ADF	100 %
Dr. Jianwei Zhao	PDF	SCDC/MII	100 %

Project Developed Materials:

1. Database of genes expressed in *B. napus* cultivars resistant and susceptible to *S. sclerotiorum*.

Supplementary Table S1. List of differentially expressed genes in *B. napus* cv. Westar
Supplementary Table S2. List of differentially expressed genes in *B. napus* cv. ZY821
Supplementary Table S3. Comparison of inoculated *B. napus* cv. ZY821 and Westar

2. *B. napus* and/or *A. thaliana* lines expressing defense genes under the control of constitutive or inducible promoter.
    - Lectin (Concanvalin A-type)
    - Lectin (Hevien-like)
    - BnPGIP1
    - BnPGIP2
    - Oxalate oxidase (alone and in combination with PGIP genes)
    - WRKY transcription factors (WRKY33, WRKY40 and WRKY53)
    - Annexin

3. Genes associated with QTLs for resistance to sclerotinia

Acknowledgements: ADF support of the project was acknowledged in the following:

Publications:

Dallal Bashi Z, Rimmer SR, Buchwaldt L, Khachatourians GG and Hegedus DD (2009) Factors affecting the concerted expression of *Sclerotinia sclerotiorum cutinaseA* and *polyglacturonase1*. (draft manuscript).

Dallal Bashi Z, Hegedus DD, Buchwaldt L, Rimmer SR and Borhan MH (2009) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene inducing peptides (NEPs). Mol. Plant Pathol – in press.

Zhao J, Buchwaldt, L, Rimmer R, Sharpe A, Bekkaoui D, Hegedus DD (2009) Temporal and qualitative differences in gene expression underly the response of resistant and susceptible *Brassica napus* cultivars to *Sclerotinia sclerotirum*. Mol. Plant Pathol. – in press.

Zhao J, Buchwaldt L, Rimmer SR, Brkic M, Bekkaoui D and Hegedus DD (2009) Differential expression of duplicated peroxidase genes in the allotetraploid *Brassica napus*. Plant Physiol. Biochem. – in press.

Hegedus DD, Li R, Buchwaldt L, Parkin I, Whitwill S, Coutu C, Bekkaoui D, Rimmer SR (2008) *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially-regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defence hormone treatment. Planta 228:241-253.

Hegedus DD and Rimmer SR (2005) *Sclerotinia sclerotiorum*: When "to be or not to be" a pathogen. FEMS Microbiology Lett. 251:177-184.

Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G and Hegedus DD (2004a) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: Cloning and characterization of

endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

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Proceedings:

Rimmer, S.R., Zhao, J., Buchwaldt, L., and Hegedus, D.D. (2007). Defining the mechanisms underlying resistance to stem rot disease (*S. sclerotiorum*) in *Brassica napus*. International Rapeseed Congress, Wuhan, China.

Conference Presentations:

Bashi, Z.D., **Hegedus, D.D.,** Rimmer, S.R. and Khachatourians, G.G. (2008) Concerted regulation of *Sclerotinia sclerotiorum cutinaseA* and *polyglacturonase1* during infection. 19th International Conference on Arabidopsis Research. Montreal, Canada.

Zhao, J., Rimmer, S.R., Buchwaldt, L., and Hegedus, D.D. (2007) Gene expression in *Brassica napus* in response to infection by *Sclerotinia sclerotiorum* and identification of WRKY transcription factors. International Congress for Arabidopsis Research, Beijing, China.

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Expense Statement: Attached

Table 1. Total number of genes that were differentially expressed in *B. napus* Zhongyou 821 and Westar stems after inoculation with *S. sclerotiorum*.

Time (h)	Zhongyou 821		Westar	
Post- Infection	Up	Down	Up	Down
6	18	0	0	0
12	286	2	54	0
24	624	245	1311	1655
48	2161	2614	2237	2597
72	2091	2304	1458	2002

Table 2. Genes up-regulated in *B.napus* Zhong You 821 stems within 6 hours after inoculation with *S. sclerotiorum*.

Oligo ID	Fold Change	P-value	*A. thaliana* locus	Description
BN24266	7.2	7.69E-09	At2g43590	chitinase
BN27647	5.2	2.32E-09	At3g16720	zinc finger (C3HC4-type) protein
BN25790	5.1	9.73E-10	At3g16530	legume lectin
BN25791	4.1	5.53E-07	At3g16530	legume lectin
BN19870	3.7	6.38E-07	At1g21100	O-methyltransferase
BN17285	3.7	9.21E-07	At2g38470	WRKY family transcription factor
BN16077	3.4	1.29E-06	At4g09030	arabinogalactan-protein (AGP10)
BN16554	3.3	1.34E-06	At1g70170	matrixin
BN24387	3.2	3.33E-06	At1g61360	S-locus lectin protein kinase
BN17170	2.8	3.21E-07	At1g14870	expressed protein
BN27200	2.8	2.35E-06	At4g11650	osmotin-like protein (OSM34)
BN14604	2.7	3.27E-09	At2g24810	thaumatin
BN15694	2.4	8.85E-06	At2g30020	protein phosphatase 2C
BN20480	2.4	1.49E-07	At5g63140	calcineurin-like phosphoesterase
BN14832	2.3	1.05E-06	At2g22500	mitochondrial substrate carrier
BN22453	2.2	4.38E-06	At1g74100	sulfotransferase
BN18515	2.1	2.05E-06	At1g32350	alternative oxidase
BN14925	2.1	1.61E-06	At3g57240	beta-1,3-glucanase (BG3)

Table 3. Functional classification of genes differentially expressed in *B. napus* Zhong You821 and Westar stems after infection with *S. sclerotiorum*.

Function*	Up-Regulated									
	ZY821					Westar				
	6	12	24	48	72	6	12	24	48	72
Cell organization and biogenesis	2	12	42	141	139	0	6	80	136	77
Developmental processes	0	7	21	72	66	0	3	47	69	40
DNA or RNA metabolism	0	0	4	23	24	0	0	7	17	5
Electron transport or energy pathways	1	21	38	87	92	0	3	62	100	82
Other biological processes	2	28	38	105	108	0	2	90	123	83
Other cellular processes	4	105	224	661	642	0	25	410	678	446
Other metabolic processes	4	114	253	719	710	0	23	463	735	503
Protein metabolism	2	35	69	231	210	0	7	126	217	124
Response to abiotic or biotic stimulus	5	49	60	156	151	0	13	119	159	127
Response to stress	1	39	52	135	143	0	7	103	152	122
Signal transduction	0	9	19	55	49	0	3	41	56	37
Transcription	1	14	25	66	65	0	4	50	73	39
Transport	1	22	49	162	146	0	8	98	168	107
Unknown biological processes	2	48	112	495	454	0	3	269	487	312
	Down-Regulated									
Cell organization and biogenesis	0	0	14	114	118	0	0	91	142	119
Developmental processes	0	0	6	94	76	0	0	55	88	81
DNA or RNA metabolism	0	0	0	16	13	0	0	17	20	13
Electron transport or energy pathways	0	0	19	106	107	0	0	71	109	86
Other biological processes	0	0	33	165	158	0	0	111	163	138
Other cellular processes	0	0	64	726	656	0	0	466	706	567
Other metabolic processes	0	0	77	813	726	0	0	533	787	631
Protein metabolism	0	0	14	260	230	0	0	149	244	197
Response to abiotic or biotic stimulus	0	0	34	209	198	0	0	142	206	180
Response to stress	0	0	26	150	139	0	0	106	151	120
Signal transduction	0	0	6	92	84	0	0	70	96	80
Transcription	0	0	14	126	116	0	0	94	133	117
Transport	0	0	15	157	131	0	0	109	149	118
Unknown biological processes	0	0	71	651	543	0	0	424	621	495

*Genes may be assigned to more than one functional category

Figure 1. ***B. napus*** **cv. ZY821 and cv. Westar 21 days after Sclerotinia infection**

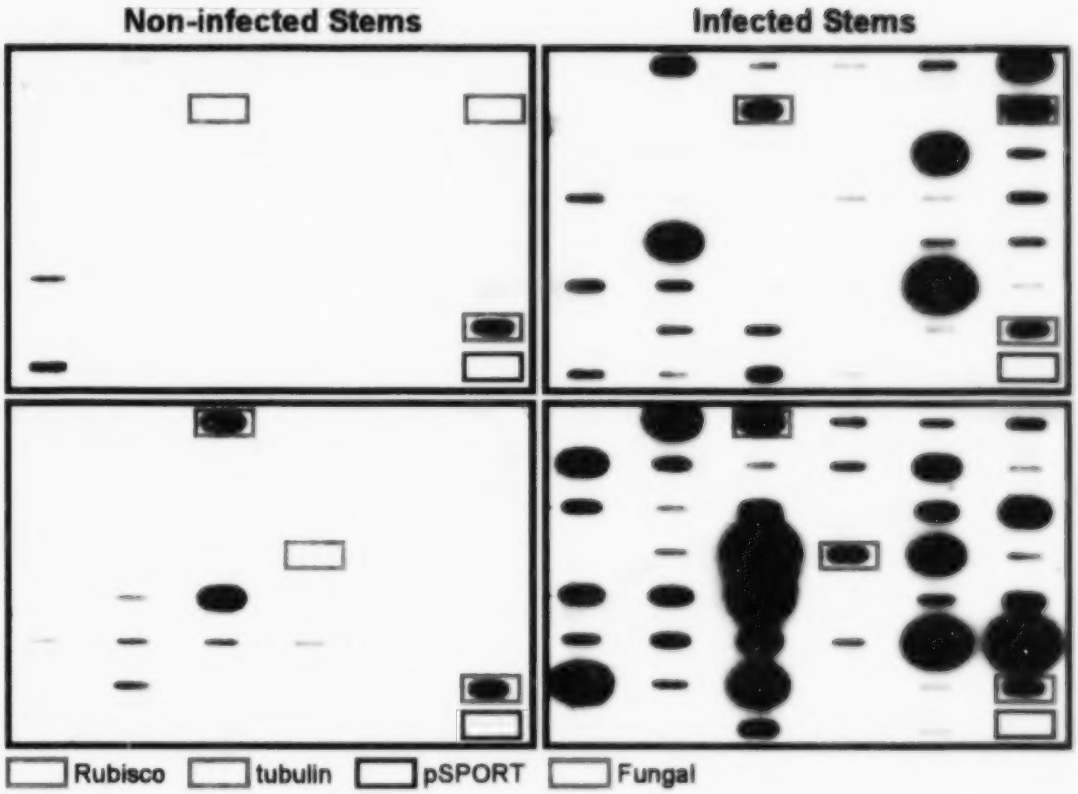

Figure 2. Reverse Northern blot analysis of a cDNA macro-array showing numerous genes expressed in *B. napus* ZY821 stems after inoculation with *S. sclerotiorum* compared to non-infected stems. Rubisco and tubulin genes are used for comparison. pSport serves as a negative control and fungal genes as positive controls for infection.

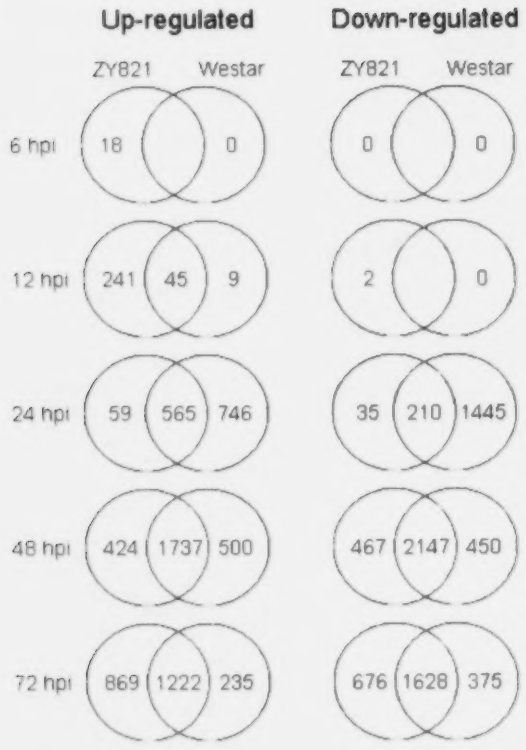

Figure 3. Venn diagrams showing the number of differentially-regulated genes that were common and unique to *B. napus* ZY821 or Westar 6 – 72 h after inoculation of stems with *S. sclerotiorum*. Genes were identified by comparing each time point using one sample t-test with a false discovery rate (FDR) of 0.005 and cut-off value of 2-fold change.

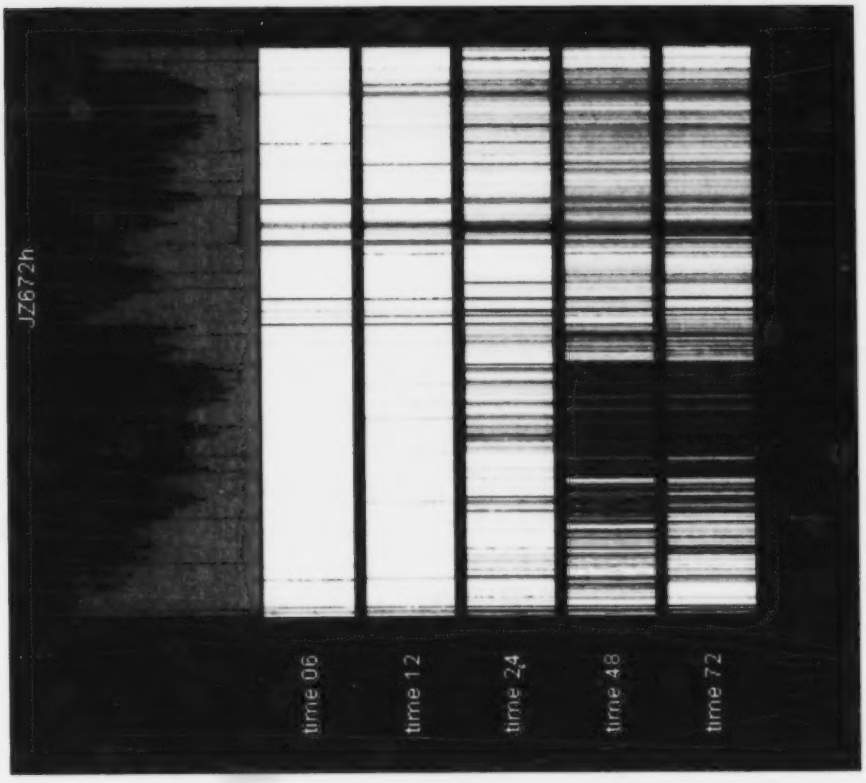

Figure 4. Dendogram showing clusters of *B. napus* ZY821 genes that were up- (red) or down- (blue) regulated at various times post-inoculation with *S. sclerotiorum*. A yellow bar indicates that no alteration in expression was observed at the specific time point. The boxed area (purple) indicates the subset of genes induced at the very early stages of the infection that are being pursued as potential candidates for developing resistance.

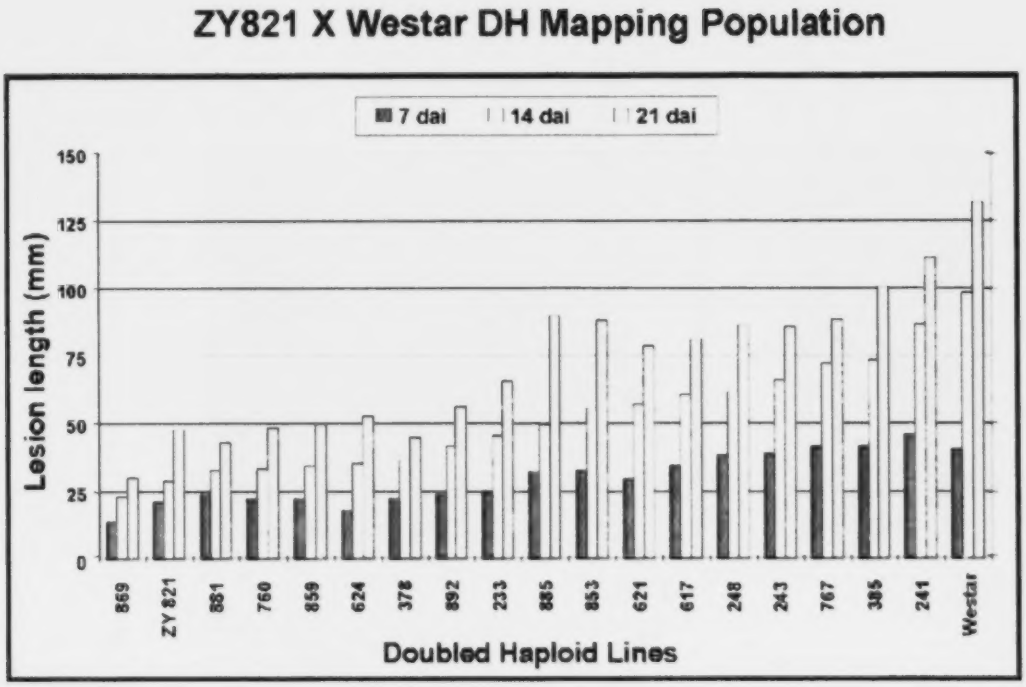

Figure 5. *S. sclerotiorum* infection of doubled haploid lines derived from a cross between the resistant *B. napus* ZY821 and the susceptible Westar.

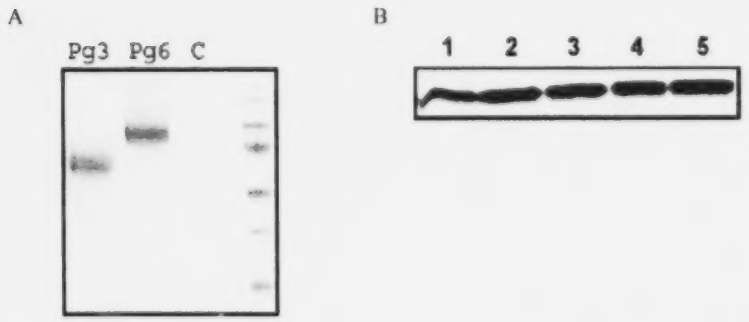

Figure 6. Expression of *S. sclerotiorum* PG3, PG6 and *B. napus* PGIP1. A; SDS-PAGE gel showing the expression of PG3 and PG6 in comparison to media from *Pichia pastoris* transformed with empty pPicz plasmid. B; western blot showing the expression of PGIP1 over a period of five days.

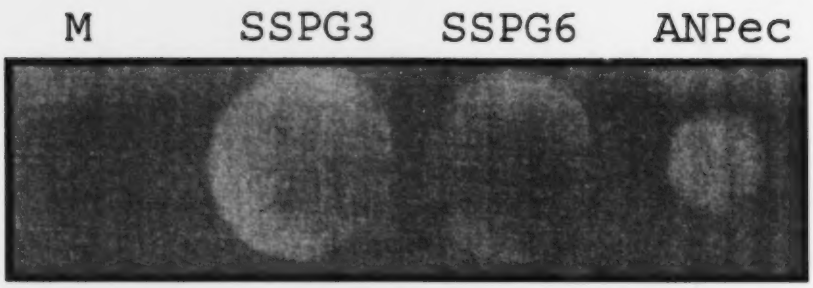

Figure 7. Pectin-agarose plate assay. 50 ul droplets containing the media from *Pichia pastoris* transformed with empty pPICZa (M), pPICZa+*Sspg3* (SSPG3) or pPICZa+*Sspg6* (SSPG6) were placed on pectin-agar plate. Commercially obtained *Aspergillus niger* pectinase (ANPec)was used as a positive control.

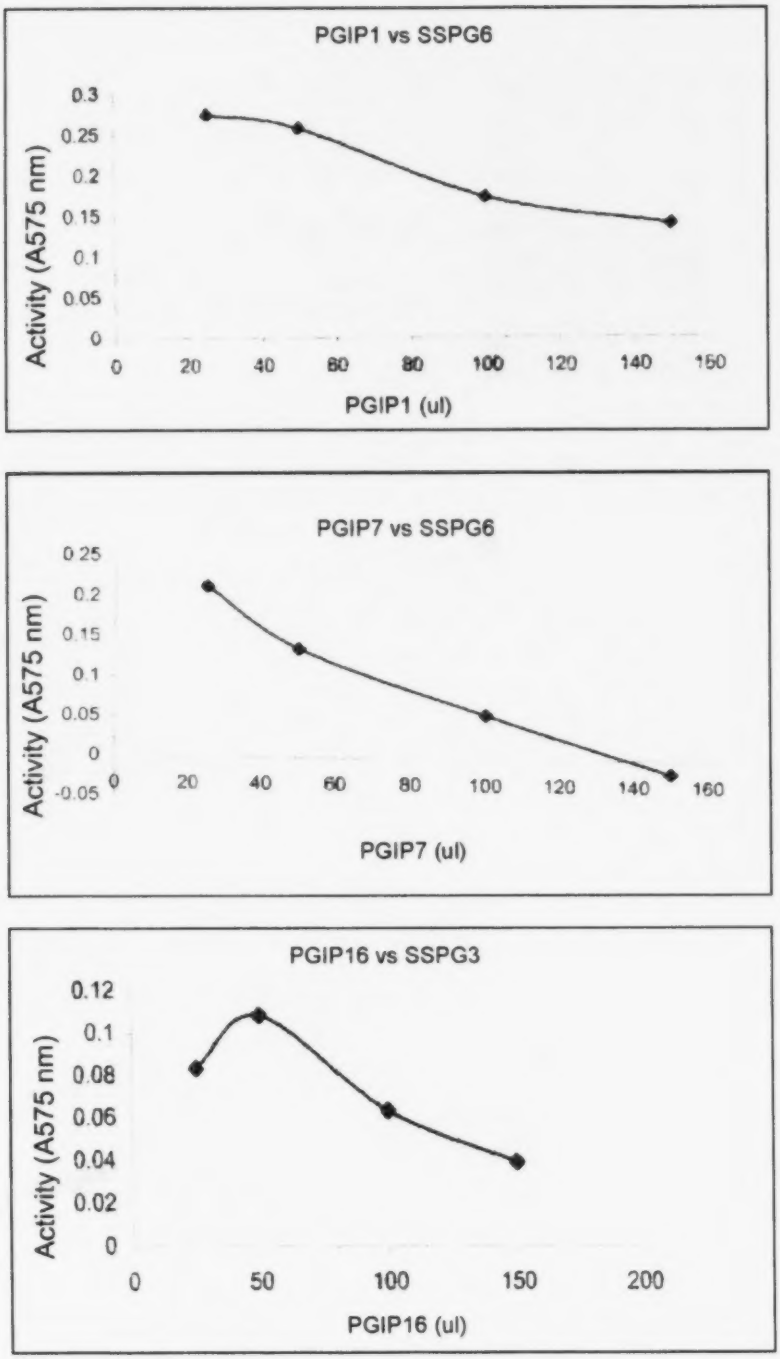

Figure 8. Inhibition of *S. sclerotiorum* SSPG activity by *B. napus* PGIP.

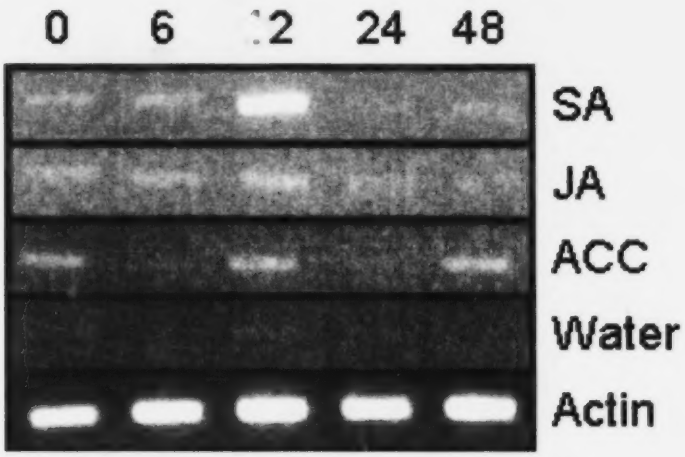

Figure 9. Effect of exogenous salicylic acid (SA), jasmonic acid (JA) and ethylene precursor (ACC; 1-aminocyclo-propane-1-carboxylic acid) treatment on BnWRKY33 gene expression in leaves at various times (hours) after application. Control leaves were sprayed with water. Amplification of the *B. napus Actin* gene was used as an RNA control for each sample, although only one is shown.

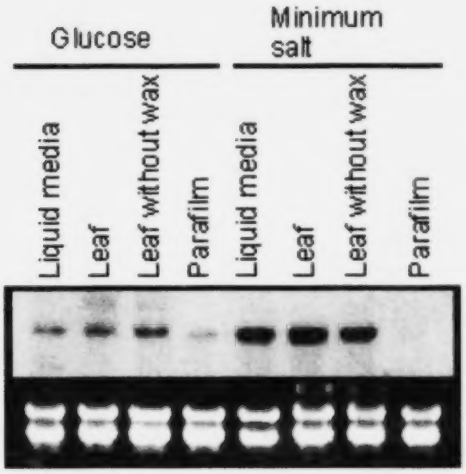

Figure 10. Expression of *Bmp1* after transfer to various surfaces. Mycelia were grown in 1% Glucose-Minimum Salt medium for 72 hours, washed with either MS or 1% Glu as indicated and then transferred to different surfaces for 24 hours.

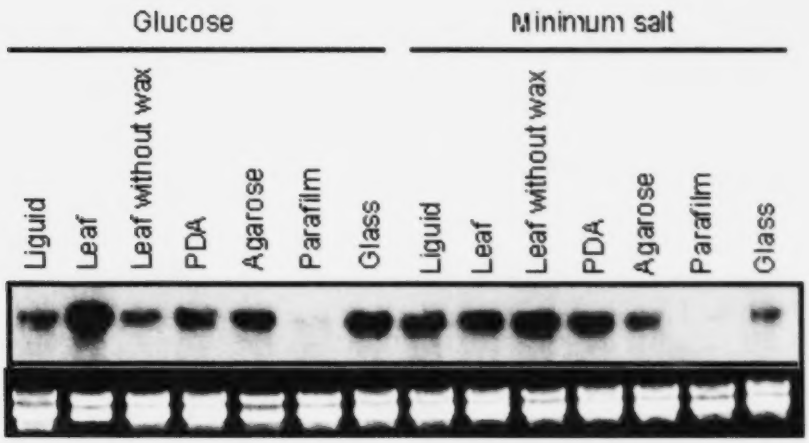

Figure 11. Expression of *Mkc1* after transfer to various surfaces. Samples were grown in 1% Glucose-Minimum Salt medium for 72 hours, washed with either MS or 1% Glu, as indicated, and then transferred to different surfaces for 24 hours.